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Mechanisms of Chloroform and Carbon Tetrachloride Toxicity in Primary Cultured Mouse Hepatocytes

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Mechanisms of chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) toxicity to primary cultured male B6C3F1 mouse hepatocytes were investigated. The cytotoxicity of both $CHCl_3$ and CCl_4 was dose- and duration-dependent. Maximal hepatocyte toxicity, as determined by lactate dehydrogenase leakage into the culture medium, occurred with the highest concentrations of $CHCl_3$ (5 mM) and CCl_4 (2.5 mM) used and with the longest duration of treatment (20 hr). CCl_4 was approximately 16 times more toxic than $CHCl_3$ to the hepatocytes. The toxicity of these compounds was decreased by adding the mixed function oxidase system (MFOS) inhibitor, SKF-525A (25μM) to the cultures. The addition of diethyl maleate (0.25 mM), which depletes intracellular glutathione (GSH)-potentiated $CHCl_3$ and CCl_4 toxicity. The toxicity of $CHCl_3$ and CCl_4 could also be decreased by adding the antioxidants *N,N'*-diphenyl-*p*-phenylenediamine (DPPD) (25 μM), α-tocopherol acetate (Vitamin E) (0.1 mM), or superoxide dismutase (SOD) (100 U/mL) to the cultures. These results suggest that: in mouse hepatocytes, both $CHCl_3$ and CCl_4 are metabolized to toxic components by the MFOS; GSH plays a role in detoxifying those metabolites; free radicals are produced during the metabolism of $CHCl_3$ and CCl_4; and free radicals may be important mediators of the toxicity of these two halomethanes.

Introduction

Chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4) are formed as by-products of sewage and drinking water chlorination and have been identified in public drinking water (*1*). Both compounds are toxic to mammalian liver *in vivo* and to isolated liver cells, with CCl_4 being more toxic than $CHCl_3$ (*2*). CCl_4 is hepatocarcinogenic in both rats and mice (*3*), whereas $CHCl_3$ has been shown to be hepatocarcinogenic only in the B6C3F1 strain of mouse when administered by corn oil gavage (*4*). Both compounds may be functioning as liver tumor promoters (*5*).

The metabolism of CCl_4 and $CHCl_3$ may provide clues to their relative cytotoxic potency and their capability as tumor promoters. It is becoming evident that free radicals generated during xenobiotic metabolism play a role in cellular toxicity (*6*), abnormal growth control (*7*), and tumor promotion (*8*). The metabolism of CCl_4 and $CHCl_3$ has been extensively studied in both rats and mice. The first step in CCl_4 metabolism is a one-electron reduction and homolytic cleavage catalyzed by cytochrome P450 of the mixed function oxidase system (MFOS) to yield the trichloromethyl radical (*9*) [Eq.(1)].

$$CCl_4 + e^- \rightarrow \cdot CCl_3 + Cl^- \quad (1)$$

P450 is maintained in the reduced form by reduced nicotinamide adenine dinucleotide (NADPH). The $\cdot CCl_3$ radical rapidly reacts with molecular oxygen to form the trichloromethylperoxyl radical (*10*) [Eq. (2)].

$$\cdot CCl_3 + O_2 \rightarrow \cdot OOCCl_3 \quad (2)$$

The $\cdot OOCCl_3$ radical is more electrophilic than the $\cdot CCl_3$ radical and may be more responsible for attacks on unsaturated fatty acids, leading to lipid peroxidation (*10*). $\cdot CCl_3$ may be more involved in covalent binding reactions of CCl_4 (*10*).

$CHCl_3$ is first metabolized by a cytochrome P450-catalyzed hydroxylation to trichloromethanol (CCl_3OH), which spontaneously dehydrochlorinates to the toxic component, phosgene ($COCl_2$) (*11*) [Eq. (3)].

$$CHCL_3 \rightarrow [CCl_3OH] \rightarrow COCl_2 + HCl \quad (3)$$

Phosgene can covalently bind to macromolecules if not detoxified by conjugation with glutathione (GSH), or

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can be further metabolized to carbon dioxide (CO_2) by reaction with water (*11*). It has also been suggested that free radicals are generated during $CHCl_3$ metabolism and that they play a role in $CHCl_3$ toxicity (*12, 13*). Most studies, however, have failed to detect enhanced levels of free radicals or of lipid peroxidation, although no study has used the B6C3F1 strain of mouse.

To provide evidence that the hepatotoxicity of $CHCl_3$ and CCl_4 in B6C3F1 mice might be caused by free radical production during the metabolism of these compounds, the present study was undertaken. We examined whether the modifiers of hepatocyte xenobiotic metabolism, SKF-525A and diethyl maleate (DEM), and the antioxidants, α-tocopherol acetate (vitamin E), *N,N'*-diphenyl-*p*-phenylenediamine (DPPD), and superoxide dismutase (SOD), could alter the toxicity of $CHCl_3$ and CCl_4 to isolated B6C3F1 mouse hepatocytes. SKF-525A is an inhibitor of the MFOS, DEM decreases intracellular GSH levels, Vitamin E and DPPD are free-radical scavengers, and SOD dismutates superoxide radicals to hydrogen peroxide.

Materials and Methods

Animals

Six-month-old male B6C3F1 mice were used in this study. They were bred at the Medical College of Ohio from C3H/He sires and C57Bl/6 dams (Charles River Laboratories, Inc., Wilmington, MA). Mice were kept in plastic cages (five per cage) containing corncob bedding and given water and certified Purina Lab Chow *ad libitum*.

Chemicals

Leibovitz's L-15 medium and gentamicin were purchased from Grand Island Biological Co. (Grand Island, NY); fetal bovine serum, from Hyclone Laboratories (Logan, UT); $CHCl_3$ and CCl_4, from Aldrich Chemical Co. (Madison, WI); and DEM, α-tocopherol acetate, DPPD, SOD, dimethyl sulfoxide (DMSO), dexamethasone and glucose, from Sigma Chemical Co. (St. Louis, MO). SKF-525A was a gift from Smith, Kline and French Laboratories (Philadelphia, PA). All other reagents were purchased from Sigma Chemical Co.

Hepatocyte Isolation and Culture

Mouse hepatocytes were isolated by two-stage *in situ* perfusion (*14*). Cell viability of the isolated cells was 90%-95%, as determined by exclusion of trypan blue. Hepatocytes were cultured in L-15 medium supplemented with glucose (1 mg/mL), dexamethasone (1 μM), fetal bovine serum (10%), and gentamicin (50 μg/mL). The hepatocytes were plated at a density of 0.5×10^6 cells per 60 mm dish and incubated in a humidified 100% air incubator at 37°C.

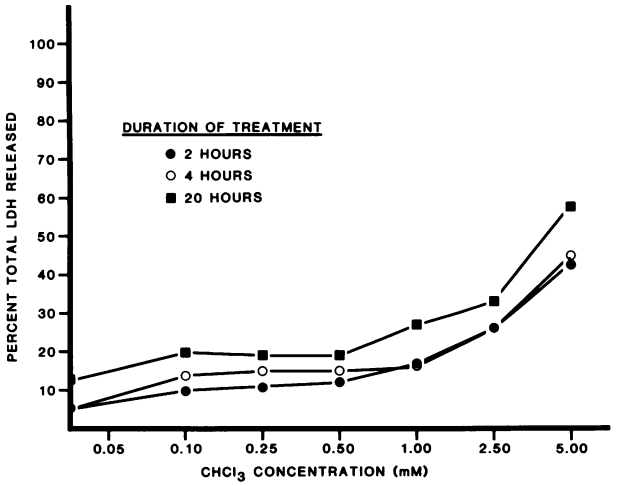

FIGURE 1. Toxicity of chloroform ($CHCl_3$) to primary cultured male B6C3F1 mouse hepatocytes after 2-, 4-, or 20-hr treatment. Each point represents the mean ($n = 3$) of the percent total LDH released.

Cytotoxicity Assay

After initial plating and a 4-hr attachment period, the cultured cells were washed once and re-fed with 5 mL of fresh medium. The cultures were then treated with the test compounds. All compounds, except SOD, were dissolved in DMSO. The final concentration of DMSO in all cultures, except for untreated cultures, was 0.4%. After 2, 4, and 20 hr of treatment, aliquots of the medium were removed, filtered through Nitrex nylon mesh to remove cells, and analyzed for lactate dehydrogenase activity (LDH) on a Beckman Multistat Analyzer (Beckman Instrument Corp., Palo Alto, CA). Total LDH per culture was also determined from 0.01% Triton X-100 lysates of untreated cultures, mean of three dishes.

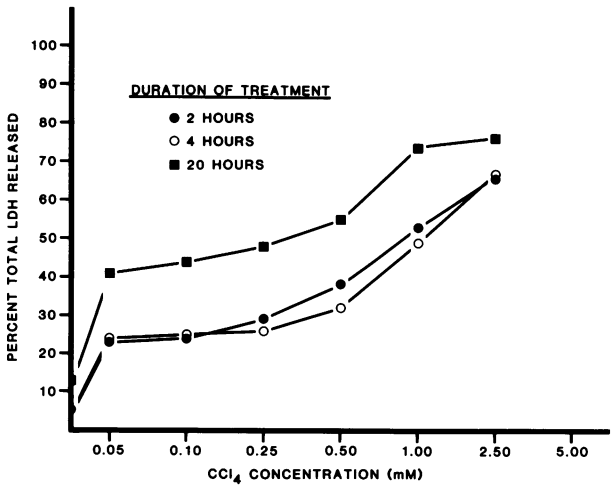

FIGURE 2. Toxicity of carbon tetrachloride (CCl_4) to primary cultured male B6C3F1 mouse hepatocytes after 2-, 4-, or 20-hr treatment. Each point represents the mean ($n = 3$) of the percent total LDH released.

Table 1. Effects of the metabolic inhibitors, SKF-525A and diethyl maleate, on chloroform toxicity in primary cultured B6C3F1 mouse hepatocytes.

Treatments		Percent total LDH release at time		
$CHCl_3$ mM	Inhibitor[a]	2 hr	4 hr	20 hr
0.0	None	5.6 ± 1.7[b]	5.7 ± 1.2	14.3 ± 2.3
0.0	SKF-525A	6.4 ± 0.9	6.8 ± 0.5	24.2 ± 1.0*
0.0	DEM	7.5 ± 1.4	7.8 ± 1.4	17.3 ± 1.3
1.0	None	16.8 ± 2.1	16.3 ± 1.9	27.0 ± 2.7
1.0	SKF-525A	12.6 ± 2.0	15.7 ± 2.4	38.9 ± 1.9*
1.0	DEM	28.3 ± 0.7*	48.8 ± 2.9*	54.4 ± 2.7*
2.5	None	25.7 ± 2.4	26.9 ± 3.9	33.3 ± 3.3
2.5	SKF-525A	15.8 ± 5.2*	19.7 ± 3.0	52.2 ± 1.8*
2.5	DEM	47.0 ± 0.6*	58.7 ± 1.8*	65.1 ± 2.0*
5.0	None	42.7 ± 5.1	44.9 ± 4.7	58.3 ± 6.0
5.0	SKF-525A	25.0 ± 0.9*	33.3 ± 0.9*	67.5 ± 2.9
5.0	DEM	57.0 ± 1.3*	65.9 ± 2.8*	73.2 ± 2.4*

[a] Inhibitor concentrations were 25 μM for SKF-525A and 0.25 μM for diethyl maleate (DEM).
[b] Mean ± SD ($n=3$).
* Significant difference versus the corresponding $CHCl_3$-only treatment.

Table 2. Effects of metabolic inhibitors, SKF-525A and diethyl maleate, on carbon tetrachloride toxicity in primary cultured B6C3F1 mouse hepatocytes.

Treatments		Percent total LDH release at time		
CCl_4 mM	Inhibitor[a]	2 hr	4 hr	20 hr
0.0	None	8.0 ± 1.0[b]	8.9 ± 1.5	14.8 ± 1.0
0.0	SKF-525A	7.9 ± 0.6	8.6 ± 0.4	24.7 ± 0.6*
0.0	DEM	7.5 ± 0.4	12.8 ± 2.4	16.3 ± 1.3
1.0	None	53.0 ± 4.8	49.3 ± 3.5	73.6 ± 2.2
1.0	SKF-525A	36.1 ± 6.9*	40.8 ± 8.5	80.7 ± 5.2
1.0	DEM	72.8 ± 2.7*	73.9 ± 4.8*	86.6 ± 0.9*
2.5	None	65.5 ± 1.6	66.8 ± 2.8	76.4 ± 5.0
2.5	SKF-525A	52.7 ± 7.7*	55.9 ± 5.1*	85.4 ± 4.6
2.5	DEM	74.4 ± 3.2*	75.5 ± 4.2*	88.0 ± 2.3*

[a] Inhibitor concentrations were 25 μM for SKF-525A and 0.25 μM for diethyl maleate (DEM).
[b] Mean ± SD ($n=3$).
* Significant difference versus the corresponding CCl_4-only treatment.

Table 3. Effects of the antioxidants DPPD, vitamin E, and superoxide dismutase on chloroform toxicity in primary cultured B6C3F1 mouse hepatocytes.

Treatments		Percent total LDH release at time		
$CHCl_3$, mM	Antioxidant[a]	2 hr	4 hr	20 hr
0.0	None	5.6 ± 1.7[b]	5.7 ± 1.2	14.3 ± 2.3
0.0	DPPD	5.1 ± 0.6	5.6 ± 0.4	11.8 ± 0.3
0.0	Vitamin E	7.3 ± 1.3	7.9 ± 0.9	16.0 ± 0.7
0.0	SOD	4.4 ± 0.3	4.7 ± 0.5	12.3 ± 1.1
1.0	None	16.8 ± 2.1	16.3 ± 1.9	27.0 ± 2.7
1.0	DPPD	9.0 ± 0.4*	5.7 ± 1.6*	14.3 ± 0.5*
1.0	Vitamin E	14.4 ± 3.5	14.8 ± 2.6	25.9 ± 3.2
1.0	SOD	9.0 ± 2.3*	13.4 ± 2.4	19.3 ± 2.6*
2.5	None	25.7 ± 2.4	26.0 ± 3.9	33.3 ± 3.3
2.5	DPPD	14.5 ± 0.9*	10.4 ± 0.2*	24.3 ± 1.9*
2.5	Vitamin E	14.0 ± 0.6*	12.5 ± 0.9*	18.6 ± 0.7*
2.5	SOD	12.1 ± 1.4*	14.3 ± 1.1*	19.7 ± 1.5*
5.0	None	42.7 ± 5.1	44.9 ± 4.7	58.3 ± 6.0
5.0	DPPD	30.3 ± 1.7*	33.2 ± 0.6*	47.5 ± 0.8*
5.0	Vitamin E	19.7 ± 2.3*	20.0 ± 2.4*	27.7 ± 2.8*
5.0	SOD	30.0 ± 4.9*	33.6 ± 2.8*	41.0 ± 2.7*

[a] Antioxidant concentrations were 25 μM for DPPD, 0.1 mM for vitamin E, and 100 U/mL for SOD.
[b] Mean ± SD ($n=3$).
* Significant difference versus the corresponding $CHCl_3$-only treatment.

Table 4. Effects of the antioxidants DPPD, vitamin E, and superoxide dismutase on carbon tetrachloride toxicity in primary cultured B6C3F1 mouse hepatocytes.

Treatments		Percent total LDH release at time		
CCl_4, mM	Antioxidant[a]	2 hr	4 hr	20 hr
0.0	None	8.0 ± 1.0[b]	8.9 ± 1.5	14.8 ± 1.0
0.0	DPPD	5.7 ± 1.5	6.2 ± 1.4	12.3 ± 1.3
0.0	Vitamin E	7.7 ± 0.6	6.9 ± 0.2	13.8 ± 0.8
0.0	SOD	7.5 ± 0.6	9.9 ± 0.8	14.8 ± 1.1
1.0	None	53.0 ± 4.8	49.3 ± 3.5	73.6 ± 2.2
1.0	DPPD	28.5 ± 3.6*	30.9 ± 5.7*	47.6 ± 4.4*
1.0	Vitamin E	40.4 ± 3.6*	34.8 ± 3.9*	60.1 ± 2.3*
1.0	SOD	36.9 ± 2.2*	37.7 ± 2.5*	58.3 ± 4.6*
2.5	None	65.5 ± 1.6	66.8 ± 2.8	76.4 ± 5.0
2.5	DPPD	44.2 ± 4.6*	50.0 ± 7.0*	61.0 ± 5.9*
2.5	Vitamin E	45.2 ± 5.2*	40.6 ± 5.7*	62.7 ± 3.9*
2.5	SOD	42.9 ± 3.0*	41.9 ± 2.1*	60.0 ± 1.4*

[a] Antioxidant concentrations were 25 μM for DPPD, 0.1 mM for vitamin E, and 100 U/mL for SOD.
[b] Mean ± SD ($n=3$).
* Significant difference versus the corresponding CCl_4-only treatment.

LDH in unused, fresh culture medium was also determined to indicate initial LDG levels in the cultures. Cytotoxicity in a culture was expressed as the "percent total LDH released" by using the formula (*15–17*):

$$\%\text{LDH} = \frac{\text{LDH in medium of treated culture} - \text{LDH in unused medium}}{\text{mean total LDH} - \text{LDH in untreated medium}} \times 100\%$$

Values for the percent total LDH released were determined in three replicate cultures for each treatment and sampling time, and the means ± S.D. were determined. Different treatments were compared statistically using Student's *t*-test.

Results

The toxicity of $CHCl_3$ to isolated B6C3F1 mouse hepatocytes was dependent on dose and treatment duration (Fig. 1). Little increase in LDH leakage was evident in cultures treated with less than 1.0 mM $CHCl_3$. CCl_4 toxicity was also dependent on dose and treatment duration (Fig. 2). As with $CHCl_3$, little difference in LDH release was evident between the 2-hr and 4-hr sampling times, and LDH release was greatest after 20 hr of treatment. CCl_4 was approximately 16 times more toxic than $CHCl_3$ to the hepatocytes. Percent total LDH activities of 50% occurred with approximately 0.25 mM CCl_4 and 4.0 mM $CHCl_3$ at the 20-hr sampling time (Figs. 1 and 2).

The addition of SKF-525A (25 μM) to $CHCl_3$- or CCl_4-treated cultures could be shown to significantly reduce LDH release below that of cultures treated only with $CHCl_3$ or CCl_4 (Tables 1 and 2). This effect was evident only at the 2-hr and 4-hr sampling times. At 20 hr, SKF-525A was cytotoxic by itself and either increased or had no effect on LDH release in $CHCl_3$- and CCl_4-treated cultures. Adding DEM (0.25 mM) to $CHCl_3$- or CCl_4-treated cultures significantly increased LDH release above $CHCl_3$- or CCl_4-only treated cultures (Tables 1 and 2). This effect was evident at 2, 4, and 20 hr. DEM was not toxic by itself.

The antioxidants, DPPD (25 μM), vitamin E (0.1 mM), and SOD (100 U/mL), when added to $CHCl_3$- and CCl_4-treated cultures, significantly reduced hepatocyte toxicity below that of cultures treated with $CHCl_3$ or CCl_4 alone (Tables 3 and 4). With the high dose of $CHCl_3$ (5 mM), vitamin E best prevented cytotoxicity compared to SOD and DPPD (Table 3). No differences in antioxidant protection were evident with 2.5 mM $CHCl_3$. DPPD provided the greatest protection against low-dose $CHCl_3$ (1 mM) toxicity. Cytotoxicity of the low CCl_4 dose (1.0 mM) was best prevented by DPPD, whereas no differences between the antioxidants were apparent with the high CCl_4 dose (2.5 mM) (Table 4).

Discussion

The results of this study indicate that $CHCl_3$ and CCl_4 are cytotoxic to primary cultured B6C3F1 mouse hepatocytes. Both compounds exhibited dose- and time-dependent effects. CCl_4 was approximately 16 times more toxic than $CHCl_3$. Previous studies have also indicated that CCl_4, is more hepatotoxic than $CHCl_3$ (*2*, *3*).

$CHCl_3$- and CCl_4-induced cytotoxicity was decreased by the simultaneous treatment of SKF-525A, an inhibitor of mammalian MFOS. These results substantiate previous studies that indicated both $CHCl_3$ and CCl_4 are metabolized in hepatocytes by the MFOS to hepatotoxic metabolites (*9*, *11*). In the case of CCl_4, the initial reaction is a reduction and homolytic cleavage of CCl_4 to the trichloromethyl radical ($\cdot CCl_3$). This radical may react directly with cellular macromolecules or may react with oxygen to form the trichloromethylperoxyl radical ($\cdot OOCCl_3$), which may then attack lipids more readily than $\cdot CCl_3$ (*10*). In any event, the initiation of free radicals and attack on membrane lipids ultimately can lead to lipid peroxidation chain reactions and cell death (*10*).

$CHCl_3$ is thought to be first hydroxylated by a P-450 reaction to trichloromethanol (CCl_3OH), which dehy-

drochlorinates spontaneously to phosgene ($COCl_2$) (*11*). Phosgene is thought to covalently bind cellular macromolecules and to be the ultimate cytotoxic component (*11*). However, two reports (*12*, *13*) have suggested that $CHCl_3$ is metabolized to free radicals and that it induces lipid peroxidation.

The results of the present study suggest that free radical production is an important mechanism of both CCl_4 and $CHCl_3$ toxicity in B6C3F1 mouse hepatocytes. The antioxidants SOD, DPPD, and vitamin E all significantly reduced the toxicity of CCl_4 and $CHCl_3$ to these cells. Similarly, depletion of cellular glutathione (GSH) (with DEM), increased CCl_4 and $CHCl_3$ toxicity to the hepatocytes. GSH functions both as an antioxidant and in the conjugation of xenobiotic metabolites (*18*).

Thus, the results of this initial study indicate that both CCl_4 and $CHCl_3$ are metabolized in B6C3F1 mouse hepatocytes by the MFOS, that cellular GSH is important in the detoxification of CCl_4 and $CHCl_3$ metabolites and/or induced free radicals, and that the cytotoxicity of both compounds might be partly mediated by free radical production. Previous studies have demonstrated the role of free radicals and lipid peroxidation in CCl_4-mediated hepatotoxicity. However, evidence that $CHCl_3$-induced hepatotoxicity is mediated by free radicals has been less substantiated. The results of the present study provide additional evidence that, in mouse hepatocytes, free radical production may be an important mechanism of $CHCl_3$-induced toxicity.

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